

# New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle

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In skeletal muscle, carnitine plays an essential role in the translocation of long-chain fatty-acids into the mitochondrial matrix for subsequent  $\beta$ -oxidation, and in the regulation of the mitochondrial acetyl-CoA/CoASH ratio. Interest in these vital metabolic roles of carnitine in skeletal muscle appears to have waned over the past 25 years. However, recent research has shed new light on the importance of carnitine as a regulator of muscle fuel selection. It has been established that muscle free carnitine availability may be limiting to fat oxidation during high intensity submaximal exercise. Furthermore, increasing muscle total carnitine content in resting healthy humans (via insulin-mediated stimulation of muscle carnitine transport) reduces muscle glycolysis, increases glycogen storage and is accompanied by an apparent increase in fat oxidation. By increasing muscle pyruvate dehydrogenase complex (PDC) activity and acetylcarnitine content at rest, it has also been established that PDC flux and acetyl group availability limits aerobic ATP re-synthesis at the onset of exercise (the acetyl group deficit). Thus, carnitine plays a vital role in the regulation of muscle fuel metabolism. The demonstration that its availability can be readily manipulated in humans, and impacts on physiological function, will result in renewed business and scientific interest in this compound.

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## Introduction

Carnitine, named from the Latin for 'flesh', was discovered in muscle tissue one hundred years ago (Gulewitsch & Krimberg, 1905) and subsequently identified as 3-hydroxy-4-*N,N,N*-trimethylaminobutyric acid, a water soluble quaternary amine, around 20 years later (Tomita & Sendju, 1927). More than 95% of the body's total carnitine store exists within skeletal muscle tissue (Brass, 1995), where it performs at least two known vital metabolic roles, the most well documented being the translocation of long-chain fatty acids (acyl groups) into the mitochondrial matrix for subsequent  $\beta$ -oxidation. Towards the end of the 20th century, a large amount of research was directed towards investigating the effects of L-carnitine supplementation on exercise performance, the main premise being that increasing carnitine availability would increase fat oxidation during prolonged exercise, spare glycogen stores and, thus, delay the onset of fatigue. However, scientific interest in L-carnitine as an ergogenic aid, and its metabolic roles, soon declined when it became apparent that L-carnitine feeding does not alter fuel metabolism during exercise or, more importantly,

impact upon the muscle carnitine pool in humans. Despite this, L-carnitine feeding as a tool to apparently promote weight loss and improve exercise performance remains the foundation of a multimillion dollar dietary supplement industry in the present day. Over the past decade, however, there has been renewed interest in the different metabolic roles of carnitine in skeletal muscle, which has led to the realization that if the muscle carnitine pool can indeed be manipulated, it can have a significant impact on physiological function.

The purpose of the present review is to outline these important, but often under-recognized, roles of carnitine in skeletal muscle fuel metabolism and to highlight how manipulating the carnitine pool of skeletal muscle, both physiologically and pharmacologically, provides insight into the regulation of muscle energy metabolism and function.

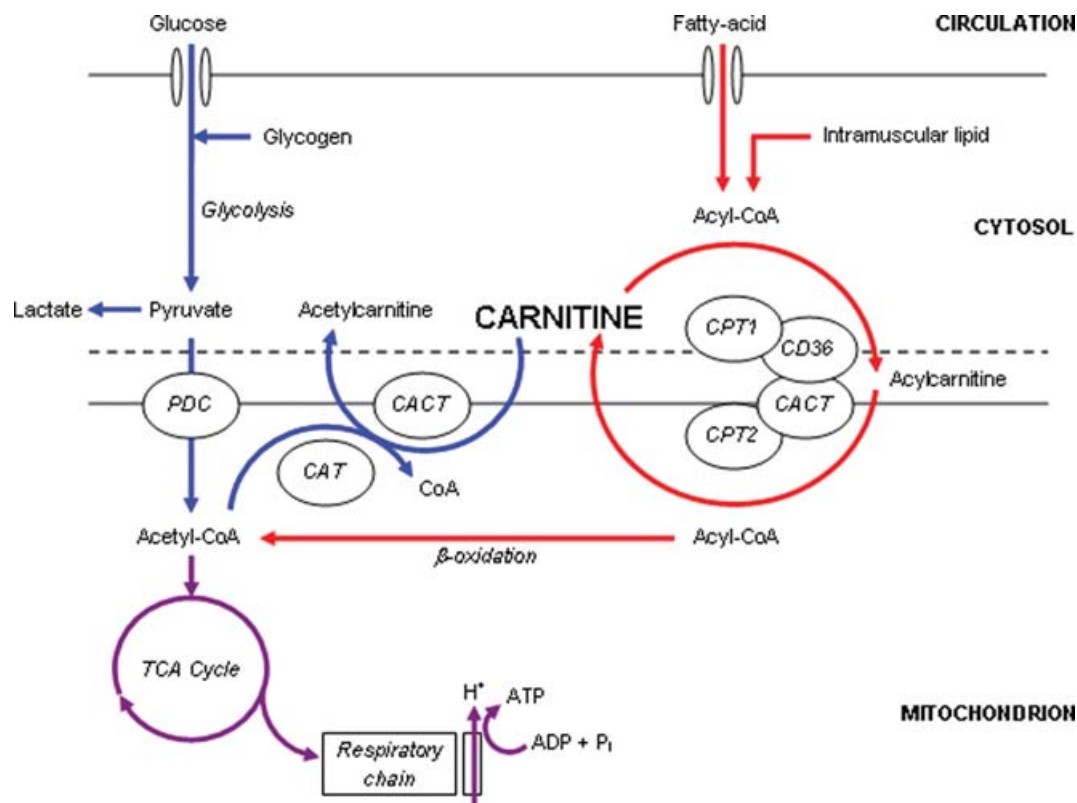
## Distinct roles for carnitine in skeletal muscle fuel metabolism

**(i) As an effector of mitochondrial long-chain fatty acyl group translocation.** Interest in carnitine as a

metabolically important compound arose in the 1950s when it was established as an essential growth factor for the *Tenebrio molitor* beetle larvae (Carter *et al.* 1952). The physiological significance of this finding became apparent over the next decade by the pioneering work of Irving Fritz, and others, which established that mitochondria in a variety of tissues are impermeable to fatty acyl-CoA, but not to fatty acylcarnitine, and that carnitine and carnitine palmitoyltransferase are essential for the translocation of long-chain fatty acids into skeletal muscle mitochondria for  $\beta$ -oxidation (Fritz, 1955; Fritz & McEwen, 1959; Bremer, 1962*a,b*; Fritz & Yue, 1963; Fritz & Marquis, 1965). Fritz and colleagues' original working hypothesis of the essential role of carnitine in the translocation of long-chain fatty acids into the mitochondrial matrix (Fritz & Yue, 1963) is represented schematically in Fig. 1, which has been modified to include more recent molecular research.

In order to take part in the  $\beta$ -oxidation pathway, cytosolic long-chain acyl-CoA must be transported across the otherwise impermeable inner mitochondrial membrane. Carnitine palmitoyltransferase 1 (CPT1), situated within the outer mitochondrial membrane

(Murthy & Pande, 1987), catalyses the reversible esterification of carnitine with long-chain acyl-CoA to form long-chain acylcarnitine. Cytosolic acylcarnitine is then transported into the mitochondrial matrix in a simultaneous 1:1 exchange with intramitochondrial free carnitine via the carnitine acylcarnitine translocase (CACT), which is situated within the mitochondrial inner membrane (Pande, 1975). Recent research involving isolated mitochondria from human skeletal muscle has suggested that the fatty acid translocase FAT/CD36, which is located in the outer mitochondrial membrane, translocates acylcarnitine from CPT1 to CACT (Bezair *et al.* 2006). Once inside the mitochondrial matrix, acylcarnitine is transesterified back to free carnitine and long-chain acyl-CoA in a reaction catalysed by carnitine palmitoyltransferase 2 (CPT2), which is situated on the matrix side of the inner mitochondrial membrane (Woeltje *et al.* 1987). The intramitochondrial long-chain acyl-CoA is then oxidized and cleaved by the  $\beta$ -oxidation pathway. CPT1 is considered to be the rate-limiting enzyme for long-chain fatty acid entry into the mitochondria and oxidation (McGarry & Brown, 1997).



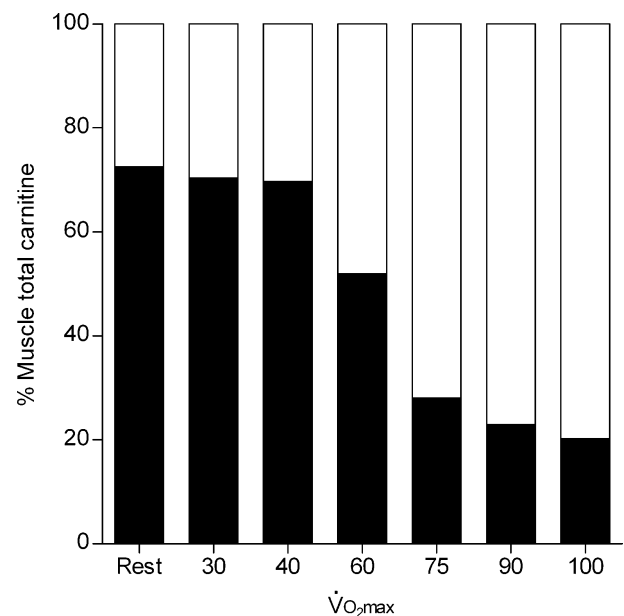
**Figure 1. A schematic diagram of the metabolic roles of carnitine in skeletal muscle**

Carnitine's role in long-chain fatty acid (acyl group) translocation into the mitochondrial matrix, for subsequent  $\beta$ -oxidation is highlighted in red, whereas the role of carnitine as a buffer of excess acetyl-CoA production is highlighted in blue. PDC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle; CAT, carnitine acetyltransferase; CACT, carnitine acylcarnitine translocase; CPT, carnitine palmitoyltransferase; CD36, fatty acid translocase.

The significance of carnitine to long-chain fatty acid oxidation is highlighted by systemic carnitine deficiency (lipid storage myopathy), where an 85% reduction in skeletal muscle carnitine content has been associated with a 75% reduction in palmitate oxidation in muscle homogenates (Engel & Angelini, 1973). Furthermore, the importance of the carnitine acyltransferase reactions is highlighted by patients with inherited enzyme deficiencies. For example, patients with CPT2 (or CACT) deficiency typically experience muscle pain and weakness, myoglobinuria, hypoglycaemia, hyperamonaemia, hypoketosis, and cardiac disorders, usually prompted by fasting, high fat intake, or prolonged exercise (Ramsay *et al.* 2001; Bonnefont *et al.* 2004). Indeed, a recent study in patients with CPT2 deficiency demonstrated that long-chain fatty acid ( $[^{13}\text{C}]$ palmitate) oxidation was normal at rest, but it was severely impaired during prolonged cycle exercise at 50% of maximal oxygen uptake ( $\dot{V}_{\text{O}_{2,\text{max}}}$ ; Orngreen *et al.* 2005). The fact that there have been no reported inherited defects in the muscle CPT1 isoform (M-CPT1), suggests that a loss of M-CPT1 might be incompatible with life.

**(ii) As an acetyl group buffer at the onset of exercise when the rate of acetyl-CoA generation is greater than its rate of utilization by the TCA cycle.** Another metabolic role of carnitine in muscle became apparent in 1966 when Childress and colleagues recognized that the blowfly flight muscle, which does not oxidize fatty acids during flight, was rich in carnitine and carnitine acyltransferase (Childress *et al.* 1966). During the initial phase of flight, acetyl-CoA was generated faster than its utilization by the TCA cycle, with a corresponding 4-fold increase in acetyl-carnitine. This led to the hypothesis that free carnitine buffers excess acetyl-CoA, which was later confirmed in contracting frog (Alkonyi *et al.* 1975) and rat (Carter *et al.* 1981) skeletal muscle, and has also been established in equine and human skeletal muscle during exercise. For example, following a few minutes of high intensity exercise, skeletal muscle free carnitine content is reduced from approximately 75% of the total muscle carnitine pool at rest ( $\sim 24 \text{ mmol (kg dry muscle)}^{-1}$  in humans) to around 20%, with almost all of the reduction being attributed to formation of acetylcarnitine (Fig. 2; Foster & Harris, 1987; Harris *et al.* 1987; Carlin *et al.* 1990; Sahlin, 1990; Constantin-Teodosiu *et al.* 1991, 1992). The increase in acetylcarnitine formation during high intensity exercise, which occurs to a greater extent in Type I muscle fibres (Constantin-Teodosiu *et al.* 1996), is directly related to an increase in muscle acetyl-CoA (Carlin *et al.* 1990; Constantin-Teodosiu *et al.* 1991), suggesting that the rate of acetyl-CoA formation from pyruvate oxidation, catalysed by the pyruvate dehydrogenase complex (PDC), is in excess of its utilization by the TCA cycle (i.e. its rate of condensation with oxaloacetate is less than its rate

of formation) leading to its subsequent accumulation. Accordingly, muscle acetylcarnitine content does not change at the onset of exercise at lower intensities (Fig. 2), suggesting that the rate of acetyl-CoA formation from pyruvate (and fatty acid oxidation) is well matched by its rate of utilization by the TCA cycle (Sahlin, 1990; Constantin-Teodosiu *et al.* 1991). Thus, during high intensity exercise (and other circumstances of increased PDC flux) carnitine buffers the excess acetyl groups formed, in a reaction catalysed by carnitine acetyltransferase (CAT), ensuring a viable pool of free CoASH is maintained for the continuation of the PDC and TCA cycle reactions (Fig. 1). Constantin-Teodosiu *et al.* (1991) calculated that if the activity of the PDC was maximal (approximately  $20\text{--}25 \mu\text{mol s}^{-1} (\text{kg wet muscle})^{-1}$ ) at high exercise intensities, and its rate of pyruvate oxidation were supported solely by the CoASH available in the muscle at rest ( $\sim 10 \mu\text{mol (kg wm)}^{-1}$ ), then the entire pool of muscle CoASH would become acetylated within 1 s of the initiation of contraction, hypothetically resulting in the immediate and complete inhibition of the PDC reaction and of the TCA cycle at the level of  $\alpha$ -ketoglutarate dehydrogenase, underlining the importance of carnitine and the rapid CAT reaction. Indeed, during the first 3 min of intense exercise ( $75\% \dot{V}_{\text{O}_{2,\text{max}}}$ ), approximately  $11\,000 \mu\text{mol (kg dm)}^{-1}$  of acetyl groups are transferred



**Figure 2.** The fraction of total carnitine ( $\sim 22 \text{ mmol (kg dry muscle)}^{-1}$ , not including long-chain acylcarnitine) represented as free (filled) or acetyl carnitine (open) in human *vastus lateralis* muscle at rest and following 4 min (or 10 min at 40, 55 and  $75\% \dot{V}_{\text{O}_{2,\text{max}}}$ ) of exercise on a cycle ergometer at various exercise intensities

Values are a review of results taken from 4 papers (Harris *et al.* 1987; Sahlin, 1990; Constantin-Teodosiu *et al.* 1991, 1992).

from the small pool of CoASH ( $\sim 45 \mu\text{mol (kg dm)}^{-1}$ ) to carnitine (Constantin-Teodosiu *et al.* 1992).

In addition to maintaining a viable pool of CoASH, the accumulation of acetylcarnitine itself provides a store of acetyl groups, which is readily available for transacetylation back to acetyl-CoA for utilization by the TCA cycle. Indeed, in the latter half of 4 h of exercise at 55%  $\dot{V}_{\text{O}_{2,\text{max}}}$ , muscle acetylcarnitine content returns to near resting values, which is paralleled by a decrease in PDC activity and therefore acetyl group delivery from pyruvate (Watt *et al.* 2002). Recent work by our group has focused on this acetyl group buffering role of carnitine in skeletal muscle by manipulating muscle PDC activation status and acetylcarnitine content, thereby providing a readily available pool of acetyl groups for utilization by the TCA cycle, which we believe is particularly important during the rest to exercise transition when inertia in mitochondrial ATP production is most evident, as will be discussed later.

### Regulation of the integration of skeletal muscle fat and carbohydrate utilization

From the research described above, it is apparent that carnitine plays a central role in both fat and carbohydrate metabolism. However, the mechanisms that regulate the relative contributions of fat and carbohydrate oxidation to skeletal muscle energy production, particularly during exercise, have not been clearly elucidated. There is increasing evidence to suggest that fatty acid oxidation is indirectly regulated by PDC flux, both at rest and during exercise, and it is apparent that the CPT1 is central to this regulation. For example, increasing glucose availability at rest, via a hyperglycaemic-hyperinsulinaemic clamp, decreases long-chain fatty acid ( $[^{13}\text{C}]$ oleate) oxidation, calculated indirectly from expired  $[^{13}\text{CO}_2]$ , with no effect on medium-chain fatty acid ( $[^{13}\text{C}]$ octanoate) oxidation (Sidossis & Wolfe, 1996). This would suggest an inhibition of fat oxidation at the level of CPT1 (medium-chain fatty acids are oxidized independent of CPT1), particularly as there was a decrease in muscle long-chain acylcarnitine content. The same inhibitory effect of carbohydrate metabolism on fat oxidation at the level of CPT1 is seen during exercise at 50%  $\dot{V}_{\text{O}_{2,\text{max}}}$ . Hyperglycaemia (induced as a result of pre-exercise ingestion of glucose) increased glycolytic flux and reduced long-chain fatty acid ( $[1-^{13}\text{C}]$ palmitate) oxidation (Coyle *et al.* 1997), whereas  $[1-^{13}\text{C}]$ octanoate oxidation was unaffected. It should be noted that these responses might have been mediated, at least in part, via an insulin-induced inhibition of adipose tissue lipolysis, resulting in decreased plasma free fatty acid (FFA) availability. However, restoring plasma FFA concentration during exercise, via lipid and heparin infusion, increased the rate of fat oxidation from 3.1 to  $4 \mu\text{mol kg}^{-1} \text{min}^{-1}$ , but did not fully restore it compared

to control ( $6.1 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ), suggesting that the inhibitory effect of increased carbohydrate availability on fat oxidation does not reside at the level of FFA availability (Horowitz *et al.* 1997). In support of this theory, reducing pre-exercise muscle glycogen content increases fat oxidation rates 2-fold during exercise at 65%  $\dot{V}_{\text{O}_{2,\text{peak}}}$ , but has no effect on the rate of muscle FFA uptake or FAT/CD36 (the protein responsible for FFA uptake into skeletal muscle) protein levels (Roepstorff *et al.* 2005). Moreover, simply increasing exercise intensity, from moderate to high, or increasing carbohydrate availability during exercise, increases PDC flux with a corresponding decrease in long-chain fatty acid oxidation rates (Romijn *et al.* 1995; van Loon *et al.* 2001; Roepstorff *et al.* 2005).

The two principal intracellular mechanisms that have been proposed to explain how increased PDC flux can lead to a down-regulation of long-chain fatty acid oxidation at the level of CPT1 are outlined below. However, it should be acknowledged that, in addition to this effect of carbohydrate flux on long-chain fatty acid oxidation, muscle pH has been proposed as a mechanism for directly regulating CPT1 activity, and thereby long-chain fatty acid oxidation during exercise. *In vitro* studies in isolated, intact mitochondria from resting human skeletal muscle have demonstrated that a decrease in pH, from 7.0 to 6.8 (which can be achieved *in vivo* during maximal exercise; Sahlin *et al.* 1976; Harris *et al.* 1977), resulted in a 50% decrease in the maximal activity of CPT1 (Starritt *et al.* 2000). However, whether a fall in muscle pH influences CPT1 activity, or fat oxidation rates, *in vivo* during exercise, particularly at submaximal exercise workloads when muscle pH is unlikely to decline, has not been determined.

#### (i) PDC flux dictates CPT1 activity via malonyl-CoA.

Malonyl-CoA is a potent inhibitor of CPT1 activity *in vitro* and is therefore a likely candidate as the intracellular regulator of the rate of long-chain fatty acid oxidation in human skeletal muscle. Indeed, in resting human skeletal muscle, changes in malonyl-CoA concentration have occurred with opposite changes in fat oxidation (Bavenholm *et al.* 2000; Rasmussen *et al.* 2002). Skeletal muscle malonyl-CoA concentration is regulated by AMP-activated protein kinase (AMPK) and by the cytosolic concentration of citrate, which, respectively, inactivate and activate the enzyme responsible for malonyl-CoA synthesis from acetyl-CoA (acetyl-CoA carboxylase; ACC; Saha *et al.* 1995). Thus, an increase in the production of acetyl-CoA and citrate, due to increased glycolytic flux, would increase muscle malonyl-CoA concentration (via an increase in ACC activity) and therefore inhibit long-chain fatty acid oxidation via CPT1. In support of this theory, a significant negative correlation was obtained between muscle malonyl-CoA

content and fat oxidation rates in healthy middle-aged men during a two-step euglycaemic–hyperinsulinaemic clamp (Bavenholm *et al.* 2000). Furthermore, hyperglycaemia with hyperinsulinaemia increased resting skeletal muscle malonyl-CoA content 3-fold ( $0.13\text{--}0.35\ \mu\text{mol (kg wet muscle)}^{-1}$ ) in healthy humans, resulting in a functional decrease in CPT1 activity (suggested by an 80% inhibition of leg [ $^{13}\text{C}$ ]oleate oxidation, with no change in [ $^{13}\text{C}$ ]octanoate oxidation), and a shunting of long-chain fatty acids towards storage (Rasmussen *et al.* 2002). However, it appears that muscle malonyl-CoA concentration may not regulate long-chain fatty acid oxidation during exercise, despite high PDC flux during exercise, and therefore the marked accumulation of acetyl groups (Constantin-Teodosiu *et al.* 1991). It has recently been demonstrated that, despite a 122% increase in fat oxidation rates (due to depleted pre-exercise muscle glycogen content) and a 2-fold increase in  $\alpha_2$ -AMPK activity during exercise at  $65\% \dot{V}_{\text{O}_{2,\text{peak}}}$ , there were no differences in muscle malonyl-CoA content, or ACC $\beta$  (muscle isoform) phosphorylation, compared to control (Roepstorff *et al.* 2005). Indeed, if anything, an increase in  $\alpha_2$ -AMPK activity would be expected to decrease muscle malonyl-CoA content. These findings are also supported by other studies where no association between malonyl-CoA content and fat oxidation rates was obtained in skeletal muscle during prolonged moderate-intensity exercise (Odland *et al.* 1996) or during graded-intensity exercise (Odland *et al.* 1998; Dean *et al.* 2000) in humans, or in rats where the time course of change in malonyl-CoA did not fit with the time course of change in substrate oxidation (Winder *et al.* 1990). Furthermore, recent evidence suggests that the sensitivity of CPT1 to malonyl-CoA paradoxically decreases in response to exercise-induced increases in whole-body fat oxidation in healthy human volunteers, and is associated with an increase in FAT/CD36 content in isolated skeletal muscle mitochondria (Holloway *et al.* 2006). It is also worth noting that there is evidence of a malonyl-CoA insensitive CPT1 isoform in rat skeletal muscle (Kim *et al.* 2002), and that phosphorylation increases the enzyme's activity and alters its sensitivity to malonyl-CoA (Kerner *et al.* 2004). Taking these findings together, it appears that malonyl-CoA does not play a major role in the regulation of CPT1 activity and therefore long-chain fatty acid oxidation in human skeletal muscle during exercise.

**(ii) PDC flux dictates free carnitine availability.** Carnitine is the principal substrate for CPT1, and therefore it may play a role in the regulation of fat oxidation during exercise. As discussed above, muscle free carnitine content declines during exercise at a high intensity (van Loon *et al.* 2001) and during moderate intensity exercise when muscle glycogen content is elevated (Roepstorff *et al.* 2005). We believe that the marked lowering of the muscle

free carnitine pool during conditions of high PDC flux may limit the ability of CPT1 to transport long-chain acyl-CoA into the mitochondrial matrix and thus the rate of fat oxidation (van Loon *et al.* 2001; Stephens *et al.* 2006a). van Loon *et al.* (2001) demonstrated that a 35% decrease in the rate of long-chain fatty oxidation that occurred at an exercise intensity above  $75\% W_{\text{max}}$ , measured using a [U- $^{13}\text{C}$ ]palmitate tracer, was paralleled by a 65% decline in skeletal muscle free carnitine content (to  $5.6\ \text{mmol (kg dm)}^{-1}$ ). More recently, Roepstorff *et al.* (2005) showed a 2.5-fold decrease in the rate of fat oxidation, compared to control, during moderate intensity exercise ( $65\% \dot{V}_{\text{O}_{2,\text{max}}}$ ) when free carnitine availability was reduced by 50% as a result of pyruvate, and therefore acetyl-CoA, production being increased as a result of pre-exercise muscle glycogen content being elevated. Also, indirect evidence has demonstrated that during bicycle exercise at  $75\% \dot{V}_{\text{O}_{2,\text{max}}}$  to exhaustion, both muscle free carnitine content and fat oxidation rates were markedly higher when pre-exercise muscle glycogen content was lowered compared to control (Putman *et al.* 1993). We have hypothesized (van Loon *et al.* 2001; Stephens *et al.* 2006a) that muscle free carnitine availability becomes limiting to CPT1 at a concentration of about  $6\ \text{mmol (kg dm)}^{-1}$  or approximately 1.8 mM intracellular water (although partitioning of free carnitine between the cytosol and the mitochondrial matrix makes it very difficult to estimate the absolute carnitine concentration in the vicinity of CPT1). This seems plausible given the reported *in vitro*  $K_m$  of CPT1 for free carnitine in human skeletal muscle is approximately 0.5 mM (McGarry *et al.* 1983). Furthermore, the catalytic site of CPT1 for carnitine is located within the contact sites of the outer mitochondrial membrane (Zammit, 1999), which may limit its exposure to the predominantly cytosolic store of free carnitine, i.e.  $\sim 90\%$  of the cellular carnitine pool (Idell-Wenger *et al.* 1978). In support of the hypothesis that free carnitine availability may limit fat oxidation, muscle free carnitine content has been shown to decrease from approximately 11 to below  $5.5\ \text{mmol (kg dm)}^{-1}$  (assuming a total carnitine content of  $22\ \text{mmol (kg dm)}^{-1}$ ) between the exercise intensities of 60 and  $\sim 80\% \dot{V}_{\text{O}_{2,\text{max}}}$  (Fig. 2), and it has been calculated (from respiratory exchange measurements) that maximal and minimal fat oxidation rates during exercise are achieved at exercise intensities of  $\sim 65$  and  $> 80\% \dot{V}_{\text{O}_{2,\text{max}}}$ , respectively (Achten & Jeukendrup, 2004).

## Manipulating the carnitine pool of skeletal muscle

**(i) Insensitivity of skeletal muscle to increased carnitine delivery.** In keeping with the hypothesis that muscle free carnitine availability can limit fat oxidation during conditions of high PDC flux, it is our premise that increasing muscle total carnitine content could

potentially alleviate the decline in fat oxidation rates routinely observed during high intensity exercise, and concomitantly reduce muscle glycogen utilization. It is worth noting that trained humans, and equines, have a higher capacity to utilize fatty acids during exercise, and appear to have a higher total muscle carnitine content compared to untrained controls (Lennon *et al.* 1983; Foster & Harris, 1992). Furthermore, increasing skeletal muscle carnitine content has been reported to delay fatigue development by 25% during electrical stimulation in rat soleus muscle strips *in vitro* (Brass *et al.* 1993). In keeping with these observations, it is the foundation of a multimillion dollar dietary supplement industry that L-carnitine feeding can increase human skeletal muscle carnitine stores and fat oxidation at rest and promote weight loss.

For the latter statement to be even considered as viable, an increase in skeletal muscle carnitine content is clearly essential. The majority of the pertinent studies in healthy humans to date have, however, failed to increase skeletal muscle carnitine content via oral or intravenous L-carnitine administration (Brass, 2000; Stephens *et al.* 2006a). For example, neither feeding L-carnitine daily for up to 3 months (Barnet *et al.* 1994; Vukovich *et al.* 1994; Wächter *et al.* 2002) nor intravenously infusing L-carnitine for up to 5 h (Brass *et al.* 1994; Stephens *et al.* 2006a) had an effect on muscle total carnitine content, or indeed net uptake of carnitine across the leg (Soop *et al.* 1988). Furthermore, feeding 2–5 g day<sup>-1</sup> of L-carnitine for 1 week to 3 months prior to a bout of exercise had no effect on perceived exertion, exercise performance,  $\dot{V}_{O_{2,max}}$ , or markers of muscle substrate metabolism such as RER,  $\dot{V}_{O_2}$ , blood lactate, leg FFA turnover, and post exercise muscle glycogen content (Greig *et al.* 1987; Oyono-Enguelle *et al.* 1988; Soop *et al.* 1988; Gorostiaga *et al.* 1989; Wyss *et al.* 1990; Decombaz *et al.* 1993; Trappe *et al.* 1994; Barnett *et al.* 1994; Vukovich *et al.* 1994; Wächter *et al.* 2002). A primary reason for L-carnitine feeding failing to impact upon total muscle carnitine content, and therefore muscle energy metabolism, in humans is undoubtedly the poor bioavailability of orally administered L-carnitine (< 20% for a 2–6 g dose; Harper *et al.* 1988; Segre *et al.* 1988). However, the finding that intravenous L-carnitine administration also has no impact on muscle carnitine content suggests that muscle carnitine transport is most likely the limiting factor to muscle carnitine accumulation in healthy humans.

In contrast to the findings in humans, it appears that an increase in muscle carnitine content can be achieved following L-carnitine supplementation in animals. For example, daily intragastric administration of ~5 mg L-carnitine for 4 weeks increased soleus muscle total carnitine content by 25% in sedentary rats and 50% in trained rats, and was associated with an increase in exercise (swimming) time to exhaustion by 15 and 30%,

respectively (Bacurau *et al.* 2003). Also, feeding horses 10 g of L-carnitine daily for 5 weeks during training increased muscle carnitine content by 50% compared to control (Rivero *et al.* 2002). However, this 50% increase in muscle carnitine content (i.e. 14 mmol (kg dm)<sup>-1</sup>; Foster & Harris, 1992) equated to more than 40% of the total L-carnitine dose administered (assuming a 50% contribution of skeletal muscle to total body mass), which is highly unlikely to be achievable in humans given the poor bioavailability of orally administered L-carnitine (Harper *et al.* 1988; Segre *et al.* 1988).

**(ii) Augmenting skeletal muscle carnitine availability in humans.** Carnitine is transported into skeletal muscle against a considerable concentration gradient (> 100 fold) via a saturable, Na<sup>+</sup>-dependent, high affinity, active transport process (Rebouche, 1977). The protein responsible for carnitine transport into skeletal muscle has been identified as the novel organic cation transporter OCTN2 (Tamai *et al.* 1998). Confirmation of OCTN2 as the protein responsible for carnitine transport *in vivo*, and its physiological significance, was presented by Nezu *et al.* (1999), who were the first to demonstrate that patients with systemic carnitine deficiency (SCD) have a loss of OCTN2 carnitine transporter function. In some SCD patients, muscle carnitine content is less than 1% of normal values (Treem *et al.* 1988; Tein *et al.* 1990). In addition, the juvenile visceral steatosis (JVS) mouse, which has muscle carnitine deficiency and presents several symptoms of SCD, has also been shown to lack high-affinity carnitine transport activity in skeletal muscle and express a mutation in OCTN2 (Nezu *et al.* 1999; Tein, 2003).

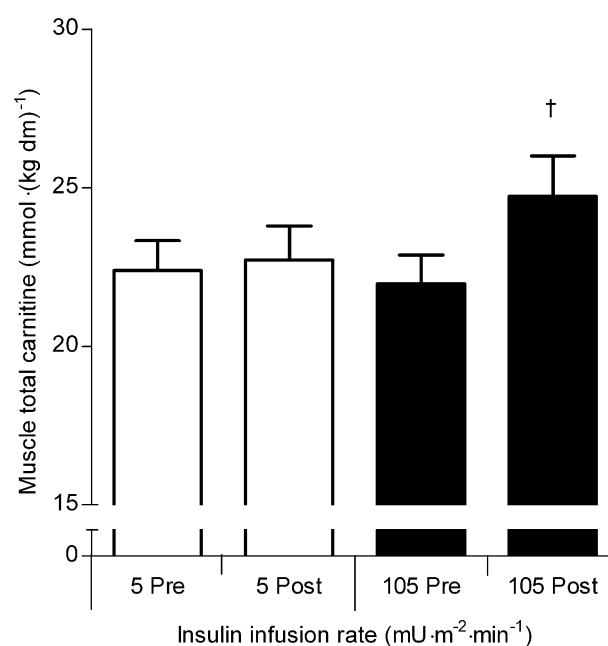
The  $K_m$  for carnitine of OCTN2, *in vitro*, is 4.3  $\mu\text{mol l}^{-1}$  (Tamai *et al.* 1998), which would suggest that in the basal state skeletal muscle carnitine uptake is saturated, and it is unlikely that plasma carnitine availability *per se* will be rate limiting to muscle carnitine transport and storage (plasma total carnitine concentration is ~50  $\mu\text{mol l}^{-1}$ ; Wachter *et al.* 2002; Stephens *et al.* 2006a,b). Indeed, maintaining a steady-state plasma carnitine concentration of ~550  $\mu\text{mol l}^{-1}$  for 5 h had no impact upon skeletal muscle total carnitine content in healthy male volunteer subjects (Stephens *et al.* 2006a; Fig. 3). However, hypercarnitinaemia (~550  $\mu\text{mol l}^{-1}$ ) combined with hyperinsulinaemia (~160 mU l<sup>-1</sup>), achieved via intravenous infusion of insulin (105 mU m<sup>-2</sup> min<sup>-1</sup> insulin clamp) and L-carnitine, increased plasma L-carnitine clearance, and resulted in an increase in skeletal muscle total carnitine content of ~15% in the same subjects (Stephens *et al.* 2006a,b; Fig. 3). Furthermore, by performing a similar experiment but with different insulin infusion rates (5, 30, 55 and 105 mU m<sup>-2</sup> min<sup>-1</sup> insulin clamps) during euglycaemic hypercarnitinaemia, it was demonstrated that plasma carnitine clearance (presumably into skeletal muscle as there was no change in

urinary carnitine excretion) is increased at a steady-state serum insulin concentration of around  $90 \text{ mU l}^{-1}$ , but not  $50 \text{ mU l}^{-1}$ , suggesting that a threshold concentration exists for the stimulatory effect of insulin on skeletal muscle carnitine accumulation (Stephens *et al.* 2006c). These findings were in concordance with our hypothesis that insulin would augment  $\text{Na}^+$ -dependent skeletal muscle carnitine transport via OCTN2, secondary to its action of increasing sarcolemmal  $\text{Na}^+/\text{K}^+$ -ATPase pump activity and therefore intracellular  $\text{Na}^+$  flux. For example, insulin increases  $\text{Na}^+/\text{K}^+$ -ATPase activity by increasing translocation of  $\alpha 2$  and  $\beta 1$  pump subunits from an intracellular storage site to the plasma membrane (Sweeney & Klip, 1998), and via an increase in the sensitivity of the  $\text{Na}^+/\text{K}^+$ -ATPase to intracellular  $\text{Na}^+$  (Clausen, 1986, 2003; Ewart & Klip, 1995). Due to the 1 : 1 stoichiometry of  $\text{Na}^+$ -carnitine cotransport via OCTN2 (Tamai *et al.* 1998), increasing  $\text{Na}^+/\text{K}^+$ -ATPase activity, via an increase in circulating insulin concentration, would lower the intracellular  $\text{Na}^+$  concentration, which would increase the electrochemical gradient for  $\text{Na}^+$  and therefore favour  $\text{Na}^+$ -carnitine cotransport. Indeed, muscle carnitine transport is inhibited by the potent  $\text{Na}^+/\text{K}^+$ -ATPase inhibitor ouabain (Rebouche, 1977; Georges *et al.* 2000), and is increased in vesicles preloaded with  $\text{K}^+$  (Berardi *et al.* 2000). In support of this hypothesis, the  $\text{Na}^+$ -dependent uptake of other nutrients, such as some amino acids (Zorzano *et al.* 2000) and creatine (Green *et al.* 1996; Steenge *et al.* 1998) by skeletal muscle, is augmented by insulin.

We have also recently demonstrated that oral feeding of L-carnitine (3 g) and carbohydrate ( $4 \times 500 \text{ ml}$  solutions each containing 94 g of simple sugars), which increased serum insulin concentration to  $\sim 75 \text{ mU l}^{-1}$ , resulted in greater whole body retention of carnitine compared to ingestion of L-carnitine alone. The retention of carnitine was calculated from measurements of 24 h urinary carnitine excretion. Furthermore, this effect was maintained throughout 2 weeks of L-carnitine ( $3 \text{ g d}^{-1}$ ) and carbohydrate feeding ( $2 \times 500 \text{ ml}$  solutions containing 94 g of simple sugars; Stephens *et al.* 2006d). Importantly, the physiologically high serum insulin concentration achieved in this experiment was in keeping with the predicted threshold, determined under insulin clamp conditions, at which an increase in muscle carnitine accumulation was thought to occur (i.e. between 50 and  $90 \text{ mU l}^{-1}$ ; Stephens *et al.* 2006c). Assuming that the greater whole-body retention of L-carnitine following daily L-carnitine and carbohydrate feeding resided within skeletal muscle (the major carnitine store within the body), this would suggest that an insulin mediated elevation of muscle total carnitine can be sustained in the long term. These findings are important, as they demonstrate that effective whole-body retention of carnitine can be achieved by L-carnitine feeding in a day-to-day setting, which is

obviously a far more practical setting than the intravenous infusion of L-carnitine and insulin.

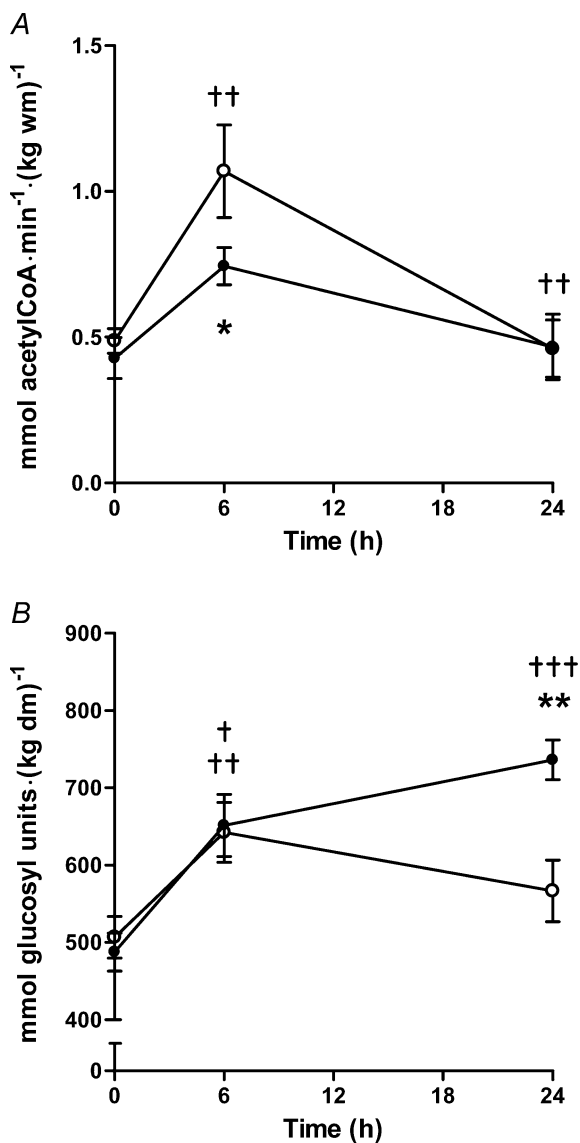
**(iii) The effect of increasing skeletal muscle free carnitine availability on muscle fuel metabolism in humans.** Our recent research (Stephens *et al.* 2006b) has demonstrated that a 15% increase in skeletal muscle carnitine content, achieved via 5 h of intravenous L-carnitine infusion during controlled hyperinsulinaemia ( $\sim 160 \text{ mU l}^{-1}$ ), resulted in a significant alteration in muscle fuel metabolism compared to control (euglycaemic hyperinsulinaemia). As would be expected, muscle PDC activity increased during euglycaemic hyperinsulinaemia under control and L-carnitine infusion conditions (Fig. 4A). However, the increase in PDC activity during L-carnitine infusion was not as pronounced as recorded in the control visit, such that post-infusion PDC activity was 30% less than control. This blunting of PDC activity following L-carnitine infusion was paralleled by a 40% reduction in muscle lactate content. Twenty-four hours after the commencement of the insulin infusion, and following an overnight fast, PDC activity and lactate content had returned to their respective basal values during both visits. However, muscle glycogen and long-chain acyl-CoA content was 30 and 40% greater than control following L-carnitine administration, respectively, despite carbohydrate administration over the previous 24 h having



**Figure 3. Muscle total carnitine content before and after 5 h of hypercarnitinaemia ( $550 \mu\text{mol l}^{-1}$ ) accompanied by intravenous insulin infusion at rates of 5 and  $105 \text{ mU m}^{-2} \text{ min}^{-1}$**

Values are the mean  $\pm$  S.E.M. ( $n = 8$ ).  $\dagger P < 0.05$ , significantly greater than pre infusion value. From Stephens *et al.* (2006a), reproduced with permission.

been exactly the same in both treatment groups (Fig. 4B). This clear difference in muscle glycogen content clearly suggests that an increase in non-oxidative glucose disposal must have occurred following L-carnitine administration.



**Figure 4**

A, muscle PDC activity before and after 5 h of intravenous saline (CON; ○) or L-carnitine (CARN; ●) infusion accompanied by a 6 h euglycaemic hyperinsulinaemic clamp (the saline/L-carnitine infusion commenced after a 1 h equilibration period), and 18 h after the end of the respective infusion visits (24 h). Values represent means  $\pm$  S.E.M. ( $n = 7$ ). †† $P < 0.01$ , significantly greater than pre CON and CARN infusion value, and significantly less than post CON and CARN infusion value. \* $P < 0.05$ , CARN significantly less than corresponding CON value. B, muscle glycogen content before and after 5 h of intravenous saline (CON; ○) or L-carnitine (CARN; ●) infusion accompanied by a 6 h euglycaemic hyperinsulinaemic clamp (the saline/L-carnitine infusion commenced after a 1 h equilibration period), and 18 h after the end of the respective infusion visits (24 h). Values represent means  $\pm$  S.E.M. ( $n = 7$ ). † $P < 0.05$ , †† $P < 0.01$ , ††† $P < 0.001$ , significantly different than pre infusion value. \*\* $P < 0.01$ , CARN significantly greater than corresponding CON value. From Stephens *et al.* (2006b), reproduced with permission; © 2006, The Endocrine Society.

Indeed, an increase in non-oxidative glucose disposal (30%), calculated indirectly as the difference between whole body glucose disposal and oxidation (measured by indirect calorimetry), during steady-state L-carnitine infusion in the presence of an elevated serum insulin concentration ( $\sim 75 \text{ mU l}^{-1}$ ), has been previously reported in other human studies (Ferrannini *et al.* 1988; Angelini *et al.* 1993; Negro *et al.* 1994). Unfortunately, the fate of the glucose was not determined in these experiments, nor were the mechanisms involved elucidated. Due to carnitine's role in long-chain fatty acyl group translocation into the mitochondrial matrix, we believe it is entirely plausible that the apparent reduction in glycolytic flux and carbohydrate oxidation outlined above (decreased PDC activity and lactate content, and increased glycogen accumulation), in the face of high carbohydrate availability, could have been caused by a carnitine-mediated increase in skeletal muscle long-chain fatty acid oxidation, i.e. an increase in long-chain acyl-CoA translocation into the mitochondrial matrix via CPT1, resulting in an increase in  $\beta$ -oxidation. According to Randle's glucose–fatty acid cycle (Randle *et al.* 1963, 1964; Garland *et al.* 1963; Garland & Randle, 1963), a concept proposed in the 1960s from experiments involving rat heart and diaphragm muscle, an increase in  $\beta$ -oxidation would result in an elevation of muscle acetyl-CoA concentration and, consequently, an increase in muscle citrate and glucose-6-phosphate content. This, in turn, would result in the down-regulation of carbohydrate flux, due to product inhibition of PDC, phosphofructokinase and hexokinase, respectively. Indeed, the decrease in PDC activity observed in our study was paralleled by a reduction in muscle lactate content and resulted in an accumulation of muscle glycogen overnight, conditions which are both consistent with the premise that glycolytic flux, and therefore carbohydrate oxidation, was inhibited. In support of this theory, muscle long-chain acyl-CoA content returned to basal overnight during the L-carnitine infusion visit (whereas it remained suppressed during the control visit), which suggests that  $\beta$ -oxidation was indeed increased (assuming that the greater long-chain acyl-CoA content observed in our study was intramitochondrial). However, as already stated, we have hypothesized that muscle carnitine availability becomes limiting to CPT1 at a value of around  $6 \text{ mmol (kg dm)}^{-1}$ , a content routinely observed in healthy humans during intense exercise. This raises the question as to why the increase in muscle carnitine content described above would increase fat oxidation in resting subjects, where free carnitine content is around  $17 \text{ mmol (kg dm)}^{-1}$  ( $\sim 5 \text{ mmol l}^{-1}$  intracellular water), well above the reported  $K_m$  of CPT1 for carnitine (0.5 mM). At present, this point requires further investigation but may be related to the compartmentalization of carnitine in skeletal muscle, which is not evident from measurements made on homogenized muscle biopsy samples. Nevertheless, taking our observations into account, it is not unreasonable



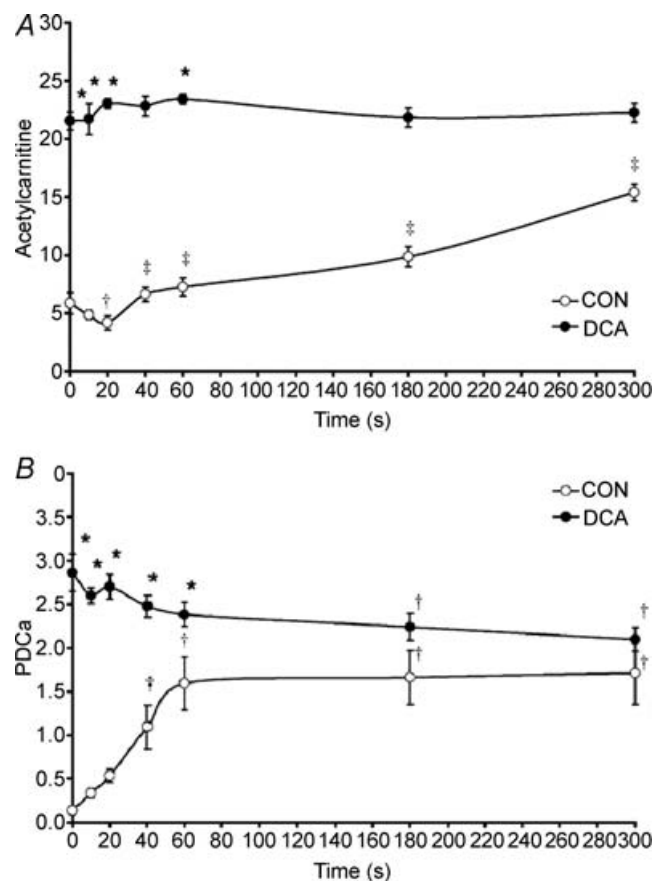
to predict that increasing muscle total carnitine content would indeed alleviate the decline in skeletal muscle fat oxidation seen during incremental exercise in healthy individuals.

**(iv) Increasing skeletal muscle PDC activity and acetyl-carnitine availability.** As outlined above, it is now well established that a lag in oxidative ATP delivery occurs at the onset of steady-state exercise (characterized by a rapid hydrolysis of phosphocreatine (PCr) and accumulation of lactate), which is attributable to inertia in mitochondrial ATP production (Yoshida *et al.* 1995; Grassi *et al.* 1998a, 1998b), rather than the classically accepted theory of a delay in muscle oxygen delivery (Margaria *et al.* 1965). However, it is currently unclear as to exactly where this mitochondrial inertia resides.

Work within our laboratory over the past decade has investigated the PDC as a potential site of limitation to mitochondrial energy production at the onset of muscular contraction. Our group was the first to demonstrate that by pharmacologically activating the PDC, using dichloroacetate (DCA; a systemic PDC kinase inhibitor), acetylcarnitine (and to a lesser extent acetyl-CoA) availability in resting skeletal muscle was markedly increased. Furthermore, PCr hydrolysis and lactate accumulation (collectively responsible for oxygen-independent ATP production) were reduced following 20 min of intense contraction under conditions of controlled muscle blood flow and oxygen delivery (Timmons *et al.* 1996). In line with this observation, it was shown that activation of the PDC at rest using DCA, which increased muscle acetylcarnitine content 10-fold, was accompanied by a 30% reduction in ATP re-synthesis from oxygen-independent routes after only 1 min of contraction (when muscle force production was identical to the control group), suggesting the existence of an inherent lag in the activation of oxygen-dependent (mitochondrial) ATP regeneration at the very onset of contraction (Timmons *et al.* 1997, 1998a,b). Following 6 min of contraction, the contribution from oxygen-independent routes to ATP re-synthesis had fallen to ~50% of that observed in the control group, while tension development was greater (Timmons *et al.* 1997). It was also apparent from these studies that some of the acetylcarnitine that was stockpiled at rest following PDC activation was utilized during the subsequent contraction (Timmons *et al.* 1996, 1997). Taking these findings together, it was concluded that the activation, and thereby flux, through PDC must limit acetyl-CoA availability and consequently mitochondrial ATP re-synthesis at the onset of exercise. Moreover, that the activation of PDC and 'priming' of mitochondria with acetylcarnitine prior to exercise, by administering DCA, could significantly increase mitochondrial ATP re-synthesis at the onset of exercise. Another important finding from this series of studies was that the onset of

fatigue development during contraction was substantially delayed following DCA administration, most probably due to the deleterious effects of PCr hydrolysis and  $P_i$  and  $H^+$  accumulation being reduced from the onset of contraction (Timmons *et al.* 1996; Timmons *et al.* 1997).

If there is indeed inertia in mitochondrial ATP re-synthesis at the onset of exercise at the level of PDC, then this would suggest that acetyl-CoA supply via the PDC will be insufficient to match the demands of the TCA cycle during this period, resulting in a reduction in the concentration of acetyl-CoA and acetylcarnitine. However, as outlined above, acetylcarnitine is thought to accumulate during moderate-to-



**Figure 5**

A, muscle acetylcarnitine concentration at rest and during 5 min of ischaemic contraction following pretreatment with saline (CON, open circles;  $n = 6$ ) or DCA (closed circles;  $n = 6$ ). Results are expressed as means  $\pm$  S.E.M. with units of mmol (kg dry muscle)<sup>-1</sup>. \*Significantly different from the corresponding CON value ( $P < 0.05$ ); †significantly lower than rest within the same group ( $P = 0.08$ ); ‡different from 20 s within same group ( $P < 0.05$ ). B, muscle pyruvate dehydrogenase complex activation status (PDCa) at rest and during 5 min of ischaemic contraction following pretreatment with saline (CON,  $\circ$ ;  $n = 6$ ) or DCA ( $\bullet$ ;  $n = 6$ ). PDCa is expressed as mmol acetyl-CoA min<sup>-1</sup> (kg wet muscle)<sup>-1</sup> at 37°C. Results are expressed as means  $\pm$  S.E.M. \*Significantly different from corresponding CON value ( $P < 0.05$ ); †significantly different from rest within the same trial ( $P < 0.05$ ). From Roberts *et al.* (2002).

intense muscular contraction (Childress *et al.* 1966; Harris *et al.* 1987; Sahlin, 1990; Constantin-Teodosiu *et al.* 1991, 1992). Thus, the hypothesis that metabolic inertia resides at the level of PDC at the onset of contraction is in contrast with the reports that acetyl-CoA production is in excess of the demands of the TCA cycle during this period. However, close scrutiny of the relevant literature reveals that most studies to date have failed to investigate the metabolic events occurring within the initial seconds of contraction, or indeed, at any time point during contraction prior to significant PDC activation. To address this issue, a study was performed in which five muscle biopsy samples were obtained from canine gracilis muscle over 1 min (rest, 10, 20, 40 and 60 s) of contraction. This study revealed that a lag in acetyl group provision (predominantly in the form of acetylcarnitine) did indeed occur during the initial 20 s of contraction (Fig. 5A), which resulted from, and was mirrored by, a lag in PDC activation (Fig. 5B). This study therefore unequivocally demonstrated the existence of a period of metabolic inertia (an 'acetyl group deficit') in skeletal muscle at the onset of contraction (Roberts *et al.* 2002).

As DCA both activates the PDC and acetylates free-coenzyme A and carnitine pools at rest (Fig. 5), it was not possible to determine from any of the above studies whether the reduction in oxygen-independent ATP re-synthesis at the onset of contraction following DCA administration was directly attributable to acetyl-CoA delivery via the PDC being increased and/or was due to a large pool of acetyl groups being made available to the TCA cycle at the immediate onset of contraction. With this question in mind, it was demonstrated that administration of sodium acetate, which markedly increased the availability of acetyl-CoA and acetylcarnitine in resting skeletal muscle, independent of PDC activation, decreased the contribution from oxygen-independent ATP production during the first minute of muscle contraction (when the PDC was largely inactive) compared to control (Roberts *et al.* 2005). However, following this first minute, when near maximal activation of PDC had occurred in both control and sodium acetate groups, it appeared that acetyl-CoA derived from the PDC, rather than stockpiled acetyl groups *per se*, was the principal route of substrate delivery to the TCA cycle. It would appear therefore that both the increase in PDC activation status and the stockpiling of acetyl groups in resting muscle as a result of DCA administration contribute to reducing inertia in mitochondrial ATP production at the onset of subsequent contraction, but the former is quantitatively more important. Similarly, it was also demonstrated that if a bout of high intensity exercise ( $75\% \dot{V}_{O_{2,max}}$ ) is performed 3 min following the performance of short duration low intensity exercise ( $55\% \dot{V}_{O_{2,max}}$ ), which increased muscle acetylcarnitine content 2-fold greater than at rest but did not affect PDC activity, this was associated with a 40%

reduction in non-oxygen-dependent ATP re-synthesis and an acceleration of  $\dot{V}_{O_2}$  on-kinetics in healthy male volunteers (Campbell-O'Sullivan *et al.* 2002). This latter observation may also partly explain the well reported ergogenic benefits of 'warming up' before intense exercise (which have classically been attributed to an exercise induced elevation of muscle temperature and/or the augmentation of local muscle blood flow).

Thus, by manipulating the muscle carnitine pool at rest, these investigations have collectively established the activation of the PDC as a rate-limiting step in the rate of rise in mitochondrial ATP re-synthesis in skeletal muscle at the onset of exercise, which in turn will dictate the magnitude of oxygen-independent ATP delivery, and thereby the rate of fatigue development during intense exercise.

### Summary and conclusions

General scientific interest in the metabolic roles of carnitine in skeletal muscle appears to have declined over recent years. However, research over the past decade has shed new light on the importance of carnitine as a regulator of skeletal muscle fuel selection and physiological function. It has been established that free carnitine availability may be limiting to the CPT1 reaction, and thus the rate of fat oxidation, during high intensity submaximal exercise when the rate of acetyl-CoA production from the PDC reaction is in excess of its rate of utilization by the TCA cycle. Furthermore, increasing muscle total carnitine content in resting healthy humans (via insulin-mediated stimulation of  $\text{Na}^+$ -dependent skeletal muscle carnitine transport) reduces muscle glycolysis, increases glycogen storage and is accompanied by an apparent increase in fat oxidation. It is important to acknowledge that an increase in human skeletal muscle carnitine content had previously not been achievable. By manipulating skeletal muscle PDC activity and acetylcarnitine availability, we have established that acetyl group delivery is limiting to mitochondrial ATP re-synthesis at the onset of exercise. Moreover, this 'acetyl group deficit' can be overcome by activating the PDC and/or stockpiling acetyl groups (mainly in the form of acetylcarnitine) at rest, which can then provide substrate to the TCA cycle from the immediate onset of intense exercise, resulting in a reduction in oxygen-independent ATP re-synthesis (PCr hydrolysis and lactate accumulation) and fatigue development.

In conclusion, manipulating the carnitine pool of skeletal muscle at rest, both physiologically and pharmacologically, has provided insight into the regulation of skeletal muscle fat and carbohydrate oxidation, both at rest and during exercise, and the interchange between anaerobic and oxidative energy provision at the onset of exercise. With the recent discovery

that the carnitine content of human skeletal muscle can be increased, a wealth of possibilities will emerge to further investigate the important roles of carnitine in skeletal muscle energy metabolism.

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